

Altered adrenal gland cholesterol metabolism in the apoE-deficient mouse

Fayanne E. Thorngate,* Penelope A. Strockbine,* Sandra K. Erickson,[†] and David L. Williams^{1,*}

Department of Pharmacological Sciences,* University Medical Center, State University of New York at Stony Brook, Stony Brook, NY 11794; and Department of Medicine,[†] University of California at San Francisco and Veterans Affairs Medical Center, San Francisco, CA 94143

Abstract Previous studies suggest the hypothesis that apoE produced by adrenocortical cells modulates cellular cholesterol metabolism to enhance the storage of esterified cholesterol (EC) at the expense of cholesterol delivery to the steroidogenic pathway. In the present study, parameters of adrenal cholesterol metabolism and corticosteroid production were examined in wild type and apoE-deficient (*apoe*^{-/-}) mice. Adrenal gland EC content and the EC/free cholesterol (FC) ratio in mice stressed by adrenocorticotropin (ACTH) treatment or saline injection were reduced in *apoe*^{-/-} compared to *apoe*^{+/+} mice. Relative to *apoe*^{+/+} mice, apoE deficiency also resulted in increased levels of plasma corticosterone in the basal state, in response to acute or long-term ACTH treatment, and after a swim-induced neuroendocrine-directed stress test. Measurements of adrenal gland scavenger receptor class B, type I (SR-BI), LDL receptor, and LDL receptor related protein (LRP) levels and the activities of ACAT or HMG-CoA reductase showed no difference between genotypes. *Apoe*^{-/-} and *apoe*^{+/+} mice showed similar quantitative increases in LDL receptors, SR-BI, adrenal weight gain, and ACAT activities in response to ACTH, and both genotypes had similar basal plasma ACTH concentrations. These results suggest that the effects of apoE deficiency reflect events at the level of the adrenal gland and are specific to changes in cholesterol accumulation and corticosterone production. Further, these findings support the hypothesis that apoE acts to enhance adrenocortical EC accumulation and diminish corticosterone production.—Thorngate, F. E., P. A. Strockbine, S. K. Erickson, and D. L. Williams. Altered adrenal gland cholesterol metabolism in the apoE-deficient mouse. *J. Lipid Res.* 2002. 43: 1920–1926.

Supplementary key words lipoprotein • steroidogenesis • corticosterone • scavenger receptor class B type I • SR-BI • LDL receptor

Apolipoprotein E (apoE) is a prominent component of plasma lipoproteins and serves to mediate endocytic uptake of remnant lipoproteins by members of the LDL re-

ceptor family (1). In contrast to other apolipoproteins, apoE is expressed in many peripheral tissues including brain, adipose, skin, kidney, lung, and steroidogenic organs such as adrenal gland, ovary, and testis (2–8). Studies with humans, nonhuman primates, and rats show that the level of apoE gene expression in the adrenal gland is similar to that in liver and may account for as much as 0.5–1% of total tissue protein synthesis (2, 8–11). ApoE mRNA and protein are expressed in adrenocortical zona fasciculata and zona reticularis cells, the sites of glucocorticoid production and cholesteryl ester storage in the adrenal cortex (12, 13). Electron microscopic immunolocalization studies show apoE intracellularly within vesicular structures of the secretory and endocytic pathways and prominently on the plasma membrane both in cell surface microvillar channels and in non-microvillar regions of the membrane (13).

The high expression of apoE in adrenocortical cells and its pattern of regulation suggest that locally-derived apoE may facilitate the acquisition of lipoprotein cholesterol, alter cellular esterified cholesterol (EC) storage, or modulate the availability of cholesterol for steroidogenesis. For example, in rat adrenal gland, apoE expression is regulated in direct proportion to EC stores and inversely to the level of steroid production (11, 12). In studies with cultured murine Y1 adrenocortical cells, constitutive expression of human apoE leads to enhanced EC accumulation and a suppression of steroid production (14, 15). In addition, inducible expression of apoE in Y1 cells enhances both the endocytic and selective uptake of LDL EC (16).

The studies noted above support the hypothesis that local apoE production modulates cellular cholesterol metabolism to enhance the storage of lipoprotein-derived EC at the expense of cholesterol delivery to the steroidogenic

Abbreviations: ACTH, adrenocorticotropin; EC, esterified cholesterol; FC, free cholesterol; LRP, LDL receptor related protein; SR-BI, scavenger receptor class B, type I.

¹ To whom correspondence should be addressed.
e-mail: dave@pharm.sunysb.edu

Manuscript received 23 May 2002 and in revised form 5 August 2002.
Published, JLR Papers in Press, August 16, 2002.
DOI 10.1194/jlr.M200205-JLR200

pathway. In the present study, parameters of adrenal cholesterol metabolism and corticosteroid production have been examined in wild type and apoE-deficient mice. The data suggest that apoE deficiency leads to an altered disposition of adrenal cholesterol with more directed toward steroid production and less toward EC storage. These data obtained with an *in vivo* mouse model support the hypothesis that local apoE synthesis modulates cellular cholesterol metabolism.

EXPERIMENTAL PROCEDURES

Animals

ApoE-deficient (*apoE*^{-/-}) mice on a mixed C57BL/6J × 129 background were kindly provided by N. Maeda (Department of Pathology, University of North Carolina, Chapel Hill, NC) (17). The mice were bred as heterozygotes to C57BL/6J mice for four generations, and then bred to homozygosity. Control (*apoE*^{+/+}) mice were obtained in the same fashion. In several experiments, control C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME), in which case the mice were acclimated in the animal colony for at least 1 week prior to experiments. Animals were maintained on a chow diet with a 12 h dark/12 h light cycle, and all experiments were initiated at 2 h into the light cycle. The experiments reported here were carried out over a period of 3.5 years using males that ranged from 2–5 months of age. Adrenal function is subject to variation due to stress in the colony (animal care procedures) that is difficult to control perfectly over a long period of time. Thus, it is not always possible to make quantitative comparisons between experiments performed at different times. However, considerable effort was made to control for potential variables within any experiment by using 1) age matched animals, 2) by initiating experiments at 2 h into the light cycle, and 3) by alternating the handling (bleeding, sacrifice, injections) of *apoE*^{-/-} and *apoE*^{+/+} mice within an experiment. All reported comparisons are between *apoE*^{-/-} and *apoE*^{+/+} mice in the same experiment. True control values (i.e., unstressed) were obtained by killing animals within a minute upon removal from the cage. Controls in experiments involving injections or non-terminal bleeding were, by necessity, subject to the stress of the experimental protocol.

Experimental treatments

For swim stress tests, mice were bled from the retroorbital plexus into EDTA coated tubes, swum for 3 min at 5°C, rested for 17 min at room temperature, anesthetized with an intramuscular injection of 50 µl ketamine-xylazine (3:2, v/v), and bled from the heart (18). For acute adrenocorticotropin (ACTH) responsiveness, mice received an intraperitoneal injection of either 0.1 ml saline or 0.1 ml saline containing 200 mU ACTH (Acthar, Rorer Pharmaceuticals, Fort Washington, PA). Mice were anaesthetized as above and killed after 40 min. For long term ACTH treatment, mice received injections of 4 U ACTH or saline as above at 0 and 12 h. At 24 h, mice were injected with saline or 200 mU ACTH and were killed after 40 min. Plasma was prepared for corticosterone assay and adrenal glands were removed, trimmed of adipose tissue, and frozen for subsequent cholesterol measurement.

Adrenal gland cholesterol

Adrenal glands from individual mice were homogenized in 0.2 ml PBS in a ground glass tissue grinder that was rinsed with another 0.2 ml PBS. The pooled homogenate and rinse were ex-

tracted with 1.5 ml CHCl₃-MeOH (1:2, v/v) for 15 min at room temperature, centrifuged for 15 min at 1,000 g, and the supernatant was removed to a new tube. The pellet was dissolved in 0.4 N NaOH and assayed for protein by the method of Lowry (19). To the supernatant were added 0.5 ml H₂O and 0.5 ml CHCl₃, the sample was mixed, centrifuged to separate phases, and the CHCl₃ phase was removed and dried down under N₂. Free cholesterol and total cholesterol were measured with enzymatic colorimetric assays (Wako Chemical) adapted to a 96 well plate format. Cholesteryl ester was determined by difference.

Plasma corticosterone and ACTH

Blood was collected into EDTA rinsed syringes or capillary tubes, centrifuged twice to remove cells, and stored at -80°C until assay. For corticosterone measurement, plasma was mixed with 10 vol of ethanol for 10 min at room temperature, centrifuged at 10,000 g for 10 min to remove protein, and aliquots were dried and assayed for corticosterone by radioimmunoassay (Endocrine Sciences, antiserum B3-163). The distribution of corticosterone between the lipoprotein and non-lipoprotein fractions of plasma was determined after ultracentrifugation of pooled plasma samples adjusted to ρ = 1.21 gm/ml with KBr (20). The ρ > 1.21 gm/ml and the ρ < 1.21 gm/ml fractions were adjusted to equal KBr concentrations, aliquots were diluted with 10 vol of ethanol, centrifuged to remove KBr, dried, and assayed for corticosterone. Plasma ACTH was determined by ELISA using a commercial kit according to the manufacturer's protocol (Sangui Biotech, Santa Ana, CA).

Enzyme assays and Western blots

Adrenal glands from 10–20 mice were pooled and homogenized in ice cold 0.6 ml 0.1 M sucrose, 0.05 M KCl, 0.04 M KH₂PO₄, 0.03 M EDTA, 10 µg/ml aprotinin, pH 7.4. Aliquots of the homogenate were frozen in liquid N₂, and stored at -80°C for Western blot analysis. The remainder of the homogenate was centrifuged at 4°C at 12,000 g for 15 min, and the supernatant was centrifuged at 105,000 g for 45 min. The microsome pellet was rehomogenized in 0.6 ml of the above buffer, centrifuged at 105,000 g for 45 min, and the washed microsomal pellet was resuspended in 0.3 ml buffer, frozen in liquid N₂, and stored at -80°C. Liver homogenate and microsomes were prepared in the same manner except with 1 gm of liver homogenized in 5 ml. Microsomes were assayed for HMG-CoA reductase activity and ACAT as previously described (21). ACAT was measured using both endogenous and exogenous substrate.

For Western blot analysis tissue homogenates (50 µg protein) were analyzed by SDS 8% polyacrylamide gel electrophoresis followed by transfer of the proteins to a nitrocellulose membrane. Blots were probed with antibodies against the LDL receptor and the LDL receptor related protein (LRP) (21, 22) and with antibody to scavenger receptor class B, type I (SR-BI) (23). After incubation with horseradish peroxidase-conjugated secondary antibody, immunoreactive bands were visualized by enhanced chemiluminescence (Pierce) and quantified by densitometry.

Adrenal morphology and ultrastructure

For light microscopy, mice were perfused through the left ventricle with PBS followed by 4% formaldehyde in PBS. Frozen sections were stained with oil red O and lightly counterstained with hematoxylin as described (18). For electron microscopy, mice were perfused at constant pressure (110 mm Hg) through the left ventricle first with 0.5 ml 0.1 M sodium cacodylate, pH 7.4, followed by 25 ml freshly prepared 2% glutaraldehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4. Tissue preparation, staining, electron microscopy, and measurement of microvillar channels were carried out with sections from two mice

of each genotype (18). Tissue morphology was also evaluated by light microscopy using toluidine blue-stained epon sections (0.5 μ m) of adrenal gland and liver tissue.

RESULTS

Plasma and adrenal cholesterol metabolism

Previous studies indicated that adrenal apoE expression is positively correlated with adrenal EC accumulation, suggesting a potential role for apoE in the maintenance or acquisition of adrenal EC stores (11).

Accordingly, plasma and adrenal gland cholesterol concentrations were determined in *apoE*^{+/+} and *apoE*^{-/-} mice under basal, unstressed, conditions and in response to either saline or ACTH treatments. The data in **Table 1** show the expected accumulation of plasma cholesterol in the *apoE*^{-/-} mice in the basal state and in all experimental groups. Acute ACTH treatment for 40 min had no effect on plasma cholesterol in the *apoE*^{+/+} or *apoE*^{-/-} mice. Long term ACTH treatment over a 24 h period, however, elevated both plasma free cholesterol (FC) and EC in *apoE*^{+/+} mice and reduced the ratio of EC/FC compared to *apoE*^{+/+} mice in the basal state or after acute ACTH or saline treatment. A similar trend toward elevated plasma FC (15–20% increased) was seen with long term ACTH treatment of *apoE*^{-/-} mice, but this did not reach statistical significance. *ApoE*^{-/-} mice subjected to long term saline or ACTH treatments also showed a significant reduction in the ratio of EC/FC compared to *apoE*^{-/-} mice in the basal state or after acute ACTH or saline treatments.

In addition to increasing corticosteroid production, long term ACTH treatment stimulates an increase in adrenal gland wet weight. As shown in **Table 2**, long term ACTH treatment increased adrenal weight similarly in both *apoE*^{+/+} and *apoE*^{-/-} mice. No differences in adrenal weight between *apoE*^{+/+} and *apoE*^{-/-} mice were seen in any experimental group.

In basal unstressed mice, we observed no significant differences in total adrenal cholesterol or in EC between *apoE*^{-/-} and *apoE*^{+/+} mice, but *apoE*^{-/-} mice had reduced adrenal FC (74 \pm 2 vs. 63 \pm 3 μ g/mg protein, *P* < 0.01). The data in **Fig. 1** and **Table 3** compare the adrenal cholesterol responses of *apoE*^{+/+} and *apoE*^{-/-} mice to acute and long term ACTH treatments. With acute ACTH treatment, *apoE*^{+/+} mice showed an increase in adrenal total

cholesterol that was due to EC accumulation (Fig. 1A, B). In comparison to *apoE*^{+/+} mice, the *apoE*^{-/-} mice showed two differences. First, the adrenal contents of total cholesterol and EC were less in the *apoE*^{-/-} mice with both acute saline and acute ACTH treatments (Fig. 1A, B). In addition, the FC content was lower in the acute saline treated *apoE*^{-/-} mice (Table 3). Second, whereas the *apoE*^{+/+} mice showed an increase in adrenal total cholesterol and EC in response to acute ACTH, the *apoE*^{-/-} mice did not.

With long term saline or ACTH treatment, the *apoE*^{-/-} mice had significantly reduced levels of adrenal total cholesterol and EC in comparison to *apoE*^{+/+} mice (Fig. 1C, D). In addition, adrenal FC content was lower in the *apoE*^{-/-} mice after long term ACTH treatment (Table 3). In contrast to the effects of acute ACTH treatment, *apoE*^{+/+} mice treated with ACTH for 24 h showed no increase in total cholesterol or EC, but did show an increase in adrenal FC content (Table 3). *ApoE*^{-/-} mice showed a significant reduction in EC in response to long term ACTH treatment (Fig. 1C, D), as well as the increase in adrenal FC content (Table 3). As compared to the *apoE*^{+/+} mice, the EC/FC ratio (Table 3) was considerably lower in the *apoE*^{-/-} mice in all treatment groups and was reduced with long term ACTH treatment in both *apoE*^{+/+} and *apoE*^{-/-} mice. This result suggests a greater mobilization of adrenal EC stores with long term, as compared to acute, ACTH treatment. The mobilization of EC was greater in the *apoE*^{-/-} mice.

Adrenal receptor levels and enzyme activities

A variety of receptor levels were determined by Western blotting of homogenates of adrenal glands from *apoE*^{-/-} and *apoE*^{+/+} mice. As shown in **Table 4**, little or no difference was seen in the levels of SR-BI, LDL receptor, or the LRP between *apoE*^{-/-} and *apoE*^{+/+} mice in the basal state. Both genotypes showed a 3.5-fold increase in SR-BI and 1.5-fold increase in LDL receptor levels in response to ACTH treatment for 24 h. Measurements made with liver homogenates also showed no difference in receptor levels between *apoE*^{-/-} and *apoE*^{+/+} mice.

Measurements of adrenal microsomal HMG-CoA reductase activity showed little or no difference between *apoE*^{-/-} and *apoE*^{+/+} mice before or after ACTH treatment (Table 4). Similarly, no differences were noted between genotypes in reductase activity in liver microsomes. Adrenal ACAT activity with either endogenous or exogenous sub-

TABLE 1. Effect of ACTH treatment on plasma cholesterol concentration

| Treatment ^a | <i>ApoE</i> ^{+/+} | | | | <i>ApoE</i> ^{-/-} | | | |
|------------------------|------------------------------|-----------------------------|------------------------------|-------|----------------------------|------------------|-------------------|-------|
| | Total Cholesterol | Free Cholesterol | Ester Cholesterol | EC/FC | Total Cholesterol | Free Cholesterol | Ester Cholesterol | EC/FC |
| | <i>mg/dl</i> | | | | <i>mg/dl</i> | | | |
| Basal | 57.3 \pm 5.7 | 14.5 \pm 1.2 | 42.7 \pm 5.2 | 2.9 | 419 \pm 142 | 162 \pm 46 | 256 \pm 107 | 1.6 |
| Acute saline | 74.8 \pm 16.8 | 18.7 \pm 5.0 | 56.0 \pm 16.6 | 3.0 | 523 \pm 114 | 176 \pm 45 | 347 \pm 96 | 2.0 |
| Acute ACTH | 78.1 \pm 22.1 | 17.7 \pm 1.8 | 60.5 \pm 21.1 | 3.4 | 479 \pm 88 | 167 \pm 40 | 312 \pm 65 | 1.9 |
| Long term saline | 75.1 \pm 10.2 | 19.8 \pm 3.7 | 55.3 \pm 6.9 | 2.8 | 384 \pm 120 | 187 \pm 72 | 197 \pm 92 | 1.1 |
| Long term ACTH | 90.9 \pm 18.0 ^b | 27.4 \pm 5.9 ^b | 63.5 \pm 13.5 ^b | 2.3 | 450 \pm 140 | 225 \pm 79 | 225 \pm 69 | 1.0 |

^a n = 20 mice/group except for basal *apoE*^{+/+} with n = 19 and basal *apoE*^{-/-} with n = 30.

^b Differs from the saline control group at *P* < 0.02.

TABLE 2. Adrenal weight response to ACTH

| Treatment ^a | <i>ApoE</i> ^{+/+} | <i>ApoE</i> ^{-/-} |
|------------------------|----------------------------|----------------------------|
| | <i>mg/adrenal pair</i> | |
| Acute saline | 3.31 ± 0.49 | 3.11 ± 0.71 |
| Acute ACTH | 3.51 ± 0.41 | 3.37 ± 0.60 |
| Long term saline | 3.33 ± 0.49 | 3.59 ± 0.51 |
| Long term ACTH | 4.09 ± 0.48 ^b | 3.98 ± 0.63 ^b |

^a n = 20 mice/group.^b Differs from the saline control group at *P* < 0.05.

strate was similar between *apoE*^{-/-} and *apoE*^{+/+} mice and showed an approximately 2-fold increase in both genotypes in response to ACTH. No difference was noted between genotypes in ACAT activity in liver microsomes.

Plasma corticosterone accumulation

Plasma corticosterone levels were elevated in *apoE*^{-/-} mice in comparison to *apoE*^{+/+} mice in the basal state (Fig. 2A, white bar) as well as in response to both acute (Fig. 2A, solid bars) and 24 h ACTH treatments (Fig. 2B, solid bars). A trivial explanation for this difference is that a greater fraction of the plasma steroid partitions into the much larger pool of plasma lipoproteins in the *apoE*^{-/-} mice but that the levels of free corticosterone and that bound to binding globulins is the same. To test this point, plasma was pooled from stressed mice of each genotype, and separated into $\rho > 1.21$ gm/ml and $\rho < 1.21$ gm/ml fractions to measure lipoprotein-associated and lipoprotein-unassociated corticosterone. The results showed that 75% of the corticosterone was in the $\rho > 1.21$ gm/ml fraction and 25% in the $\rho < 1.21$ gm/ml fraction in both ge-

notypes (data not shown). Additionally, no significant correlation was observed between total plasma cholesterol and plasma corticosterone in either *apoE*^{-/-} or *apoE*^{+/+} mice. Thus, the elevated plasma corticosterone in *apoE*^{-/-} mice appears to reflect a physiological difference that is not explained by the much larger plasma lipid pool in these mice.

To determine whether elevated basal plasma corticosterone in *apoE*^{-/-} mice is an effect at the adrenal gland or reflects increased ACTH stimulation through the hypothalamic-hypophyseal axis, plasma ACTH concentrations were measured. No significant difference between genotypes was found (*apoE*^{+/+}, 46 ± 14 pg/ml, n = 14; *apoE*^{-/-}, 68 ± 13 pg/ml, n = 24). Additionally, plasma corticosterone accumulation in response to an acute swim stress test was determined as a measure of a central nervous system-directed adrenal response. As shown in Fig. 3, as compared to the *apoE*^{+/+} mice, corticosterone values were elevated in the *apoE*^{-/-} mice before and after the swim stress test. The fold increase in corticosterone upon stress was approximately the same in both genotypes (*apoE*^{+/+} 3.3 vs. *apoE*^{-/-} 2.6).

Adrenal morphology and HDL localization in microvillar channels

Adrenal morphology was evaluated at the light microscopic level by oil red O/hematoxylin staining of cryosections and by toluidine blue staining of thick sections of epon-embedded tissue prepared for electron microscopy. No significant differences were noted between *apoE*^{-/-} and *apoE*^{+/+} tissue (data not shown). Unlike the situation in the skin of *apoE*^{-/-} mice where macrophage foam cells

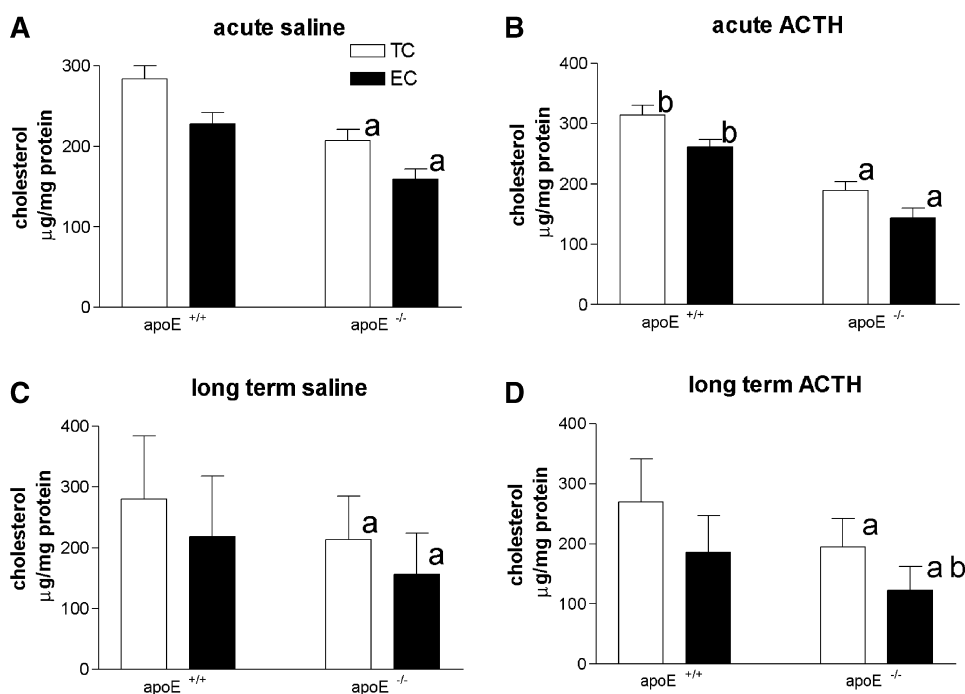


Fig. 1. Adrenal gland cholesterol content in response to adrenocorticotropin (ACTH). Mice (n = 20) were injected with saline or ACTH for either an acute (A and B) or long term (C and D) treatment as described in Experimental Procedures. Open bars, total cholesterol; solid bars, esterified cholesterol. ^a *P* < 0.02 versus respective *apoE*^{+/+} group. ^b *P* < 0.05 versus respective saline-treated group.

TABLE 3. Adrenal gland free cholesterol content in response to ACTH

| Treatment ^a | <i>ApoE</i> ^{+/+} | | <i>ApoE</i> ^{-/-} | |
|------------------------|--------------------------------------|-------|--------------------------------------|-------|
| | FC | FC/EC | FC | FC/EC |
| | adrenal cholesterol μg/mg protein | | adrenal cholesterol μg/mg protein | |
| Acute saline | 56 ± 3 | 4.1 | 48 ± 2 ^b | 3.3 |
| Acute ACTH | 53 ± 4 | 4.9 | 48 ± 4 | 3.0 |
| Long term saline | 61 ± 7 | 3.6 | 58 ± 9 | 2.7 |
| | <i>P</i> < 0.01 | | <i>P</i> < 0.01 | |
| Long term ACTH | 84 ± 18 | 2.2 | 73 ± 13 ^c | 1.7 |

^a n = 20 mice/group.

^b *P* < 0.02 versus respective *ApoE*^{+/+} value.

^c *P* values in the table refer to comparison between ACTH and saline groups.

and inflammatory cells accumulate (24), the adrenal gland showed no signs of inflammation or macrophage foam cells (data not shown). Oil red O staining appeared somewhat less dense in some adrenal glands of *ApoE*^{-/-} mice, but this difference was not consistently observed. Liver sections from *ApoE*^{-/-} mice, but not *ApoE*^{+/+} mice, showed many lipid laden Kupffer cells, granuloma-like accumulations of histiocytes in subendothelial locations, and hepatocytes containing increased numbers of lipid droplets; the latter point was noted previously (25). Adrenal ultrastructure at the electron microscopic level also appeared to be normal in *ApoE*^{-/-} mice. The width of adrenal microvillar channels (*ApoE*^{+/+}, 12.7 ± 2.3 nm, n = 51; *ApoE*^{-/-}, 13.5 ± 2.8 nm, n = 68) and appearance of HDL particles in the channels were the same in both genotypes.

DISCUSSION

This study shows significant differences in adrenal gland cholesterol metabolism in *ApoE*^{-/-} mice. The most prominent effects were observed in adrenal cholesterol content in ACTH-treated mice or mice stressed by saline injection. In both groups, adrenal EC content and the

EC/FC ratio were lower in *ApoE*^{-/-} mice, suggesting a reduced capacity to maintain adrenal cholesterol stores during stress. In some groups adrenal FC content was also reduced in *ApoE*^{-/-} adrenal glands. These results complement data from a previous study that showed increased FC and EC accumulation in murine Y1 adrenal cells that over-expressed apoE (14). Additionally, the present results complement previous studies in the rat that demonstrated a direct correlation between the levels of apoE mRNA and adrenal EC content under conditions that produced a 15-fold range of these parameters (11). Taken together with these previous studies, the present findings in the *ApoE*^{-/-} mouse support a physiological role for apoE in maintaining the EC content of adrenocortical cells. Because these changes were seen only in adrenal glands from stressed mice, apoE may act to maintain adrenal cholesterol stores in the face of increased cholesterol utilization for steroid production.

The absence of apoE also resulted in increased levels of plasma corticosterone in the *ApoE*^{-/-} mouse in the basal state, in response to acute or long-term ACTH treatment, and after a swim-induced neuroendocrine-directed stress. Increased plasma corticosterone in the basal state or in response to long-term ACTH treatment could result from increased adrenal production or reduced plasma clear-

TABLE 4. Receptor and enzyme activities in *ApoE*^{+/+} and *ApoE*^{-/-} mice

| Parameter | Adrenal Gland | | | | | | | | |
|---------------------------|----------------|----------------------------|----------------------------|------|----------------------------|----------------------------|-------|----------------------------|----------------------------|
| | Basal | | | ACTH | | | Liver | | |
| | n ^a | <i>ApoE</i> ^{+/+} | <i>ApoE</i> ^{-/-} | n | <i>ApoE</i> ^{+/+} | <i>ApoE</i> ^{-/-} | n | <i>ApoE</i> ^{+/+} | <i>ApoE</i> ^{-/-} |
| SR-BI ^b | 4 | 100 | 105 ± 32 | 4 | 337 ± 80 | 355 ± 95 | 4 | 37 ± 16 | 27 ± 9 |
| LDL receptor ^b | 8 | 100 | 139 ± 15 | 8 | 154 ± 20 | 209 ± 17 | 5 | 69 ± 11 | 72 ± 14 |
| LRP ^b | 4 | 100 | 85 ± 14 | | ND ^c | ND | 4 | 111 ± 4 | 96 ± 12 |
| HMG CoA reductase | | | | | | | | | |
| pmoles/min/mg protein | 6 | 61 ± 29 | 52 ± 18 | 3 | 20 ± 16 | 40 ± 12 | 4 | 79 ± 8 | 93 ± 26 |
| ACAT | | | | | | | | | |
| pmoles/min/mg protein | | | | | | | | | |
| Endogenous substrate | 4 | 20 ± 4 | 33 ± 8 | 3 | 48 ± 40 | 63 ± 8 | | ND | ND |
| Exogenous substrate | 7 | 400 ± 100 | 280 ± 50 | 3 | 760 ± 70 | 850 ± 50 | 4 | 250 ± 30 | 270 ± 30 |

^a n = number of homogenate or microsome preparations examined. Each preparation was derived from adrenal glands or liver samples pooled from 10–20 mice.

^b Receptor levels reflect densitometric units expressed as a percent of the basal *ApoE*^{+/+} sample set at 100.

^c Not determined.

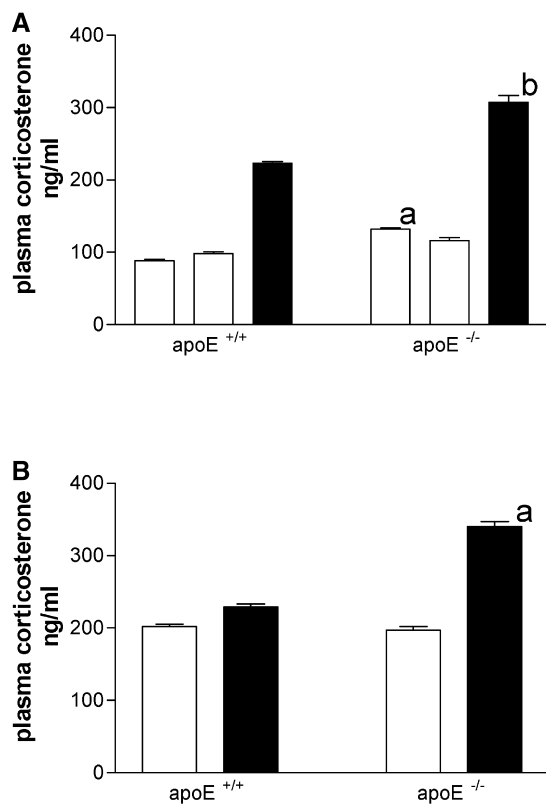


Fig. 2. Plasma corticosterone in response to ACTH. Mice were untreated (A, basal, left bar; *apoE*^{+/+} n = 18; *apoE*^{-/-} n = 31), or injected with saline (middle bar) or ACTH (solid bars) for either an acute (A, n = 20) or long term (B, n = 10) treatment as described in Experimental Procedures. ^a *P* < 0.01 versus respective *apoE*^{+/+} group. ^b *P* < 0.07 versus respective *apoE*^{+/+} group.

ance. The rapid increase in plasma corticosterone following a 3-min swim or an acute ACTH treatment, however, suggests that elevated corticosterone levels in the *apoE*^{-/-} mouse result from greater adrenal hormone production. The present results do not distinguish between an indirect systemic effect and a direct adrenal effect of the absence of apoE on steroid production. However, a direct effect of apoE on adrenocortical cell steroid production is suggested by previous studies that showed suppression of steroid production in murine Y1 adrenal cells that constitutively over-express apoE (15). Additionally, the finding that apoE mRNA concentrations and relative apoE synthesis rates are very high in adrenocortical cells of humans, non-human primates, and rodents supports a local effect of apoE (2, 4, 5, 8, 10). This interpretation of the present findings also is supported by the inverse relationship between adrenal apoE mRNA content and serum corticosteroids observed in the rat (11). The mechanism through which steroid production is increased in the *apoE*^{-/-} adrenal gland may reflect the failure of apoE to dampen responsiveness through the protein kinase A pathway and suppress expression of the mRNA for the cholesterol side chain cleavage enzyme. ApoE exhibits these effects when over expressed in cultured murine adrenal cells (26, 27).

The present results showing elevated corticosterone levels in *apoE*^{-/-} mice are in agreement with a recent study

(28), although a few differences were noted. Raber et al. (28) saw elevated corticosterone levels in 6-month-old *apoE*^{-/-} mice but not in 3-month-old mice, whereas in the present study elevated corticosterone levels were seen in 2–5-month-old *apoE*^{-/-} mice. We observed no correlation between plasma corticosterone levels and animal age in control or ACTH treated mice. Raber et al. (28) also reported increased lipid droplets in the adrenal cortex and medulla of *apoE*^{-/-} mice as judged by Nile red staining suggesting increased EC accumulation. In the present study we observed decreased EC accumulation in the adrenal glands of *apoE*^{-/-} mice by cholesterol measurement, occasional but inconsistent decreases in oil red O staining in the adrenal cortex, and no oil red O staining in the adrenal medulla. These discrepancies with Raber et al. (28) may reflect methodological differences.

Among the parameters studied, the effects of apoE deficiency are specific to adrenal cholesterol accumulation and corticosterone production. No significant differences were observed in adrenal LDL or HDL receptor levels, ACAT or HMG-CoA reductase activities, or in the response of these parameters to ACTH treatment. Similarly, adrenal weight gain in response to ACTH treatment was similar in both genotypes. Additionally, the absence of morphological differences at the light and electron microscopic levels and the absence of inflammatory cells in the adrenal glands of *apoE*^{-/-} mice suggest that the effects of apoE deficiency are relatively specific to changes in cholesterol accumulation and corticosterone production. The finding of similar basal plasma ACTH levels in the *apoE*^{-/-} and *apoE*^{+/+} mice suggests that the observed differences in adrenal cholesterol metabolism and steroid production are due to apoE effects on adrenocortical cells and not to effects on a neuroendocrine pathway. This point is also supported by the similar quantitative responses of both genotypes in the ACTH stimulation of LDL receptors, SR-BI, adrenal weight gain, and ACAT activities whereas at the same time *apoE*^{-/-} mice showed elevated plasma corti-

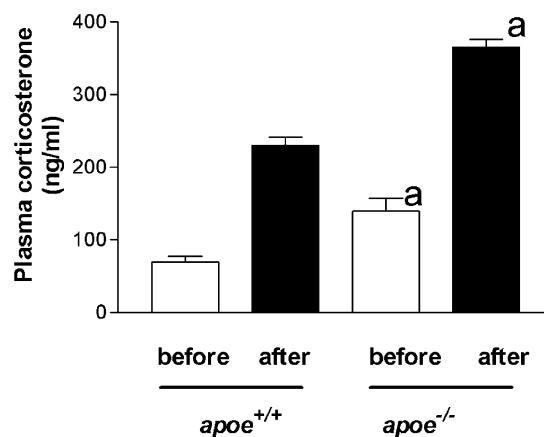



Fig. 3. Plasma corticosterone in response to a swim stress test. Plasma corticosterone was measured (n = 18) before (open bars) and after (solid bars) a swim stress test as described in Experimental Procedures. ^a *P* < 0.01 versus respective *apoE*^{+/+} group.

costerone levels and a decreased capacity to maintain adrenal cholesterol stores when stressed.

The mechanism by which apoE expression alters adrenal cholesterol metabolism is unclear but could involve changes in lipoprotein cholesterol uptake or in cholesterol trafficking or utilization in adrenocortical cells. Previous studies in Y1 adrenocortical cells showed that apoE expression increases the selective uptake of LDL EC via a pathway that does not involve SR-BI (16, 29). A smaller increase in the endocytic uptake of LDL EC was also seen upon apoE expression. Similar effects of apoE could contribute to the maintenance of adrenal gland EC during stress, although HDL, and not LDL, appears to provide most of the adrenal gland EC in vivo (18, 30). Further studies on the effect of apoE on HDL and LDL EC uptake into adrenocortical cells may be informative on this issue. 

This research was supported by Grants HL32868 and HL52069 from the National Institutes of Health and a Merit Award from the Department of Veterans Affairs. Steven Lear and Jacqueline Parton provided excellent technical assistance.

REFERENCES

1. Mahley, R. W., and Z-S. Ji. 1999. Remnant lipoprotein metabolism: key pathways involving cell-surface heparan sulfate proteoglycans and apolipoprotein E. *J. Lipid Res.* **40**: 1–16.
2. Blue, M-L., D. L. Williams, S. Zucker, S. A. Khan, and C. B. Blum. 1983. Apolipoprotein E synthesis in human kidney, adrenal gland, and liver. *Proc. Natl. Acad. Sci. USA.* **80**: 283–287.
3. Dawson, P. A., N. Schechter, and D. L. Williams. 1986. Induction of rat E and chicken A-I apolipoproteins and mRNAs during optic nerve degeneration. *J. Biol. Chem.* **261**: 5681–5684.
4. Driscoll, D. M., and G. S. Getz. 1984. Extrahepatic synthesis of apolipoprotein E. *J. Lipid Res.* **25**: 1368–1379.
5. Elshourbagy, N. A., W. S. Liao, R. W. Mahley, and J. M. Taylor. 1985. Apolipoprotein E mRNA is abundant in the brain and adrenal, as well as in the liver and is present in other peripheral tissues of rats and marmosets. *Proc. Natl. Acad. Sci. USA.* **82**: 203–207.
6. Fenjves, E. S., D. A. Gordon, L. K. Pershing, D. L. Williams, and L. B. Taichman. 1989. Systemic distribution of apolipoprotein E secreted by grafts of epidermal keratinocytes: Implications for epidermal function and gene therapy. *Proc. Natl. Acad. Sci. USA.* **86**: 8803–8807.
7. Lenich, C., B. Brecher, S. Makrides, A. Chobanian, and V. I. Zannis. 1988. Apolipoprotein gene expression in the rabbit: abundance, size, and distribution of apolipoprotein mRNA species in different tissues. *J. Lipid Res.* **29**: 755–764.
8. Williams, D. L., P. A. Dawson, T. C. Newman, and L. L. Rudel. 1985. Apolipoprotein E synthesis in peripheral tissues of nonhuman primates. *J. Biol. Chem.* **260**: 2444–2451.
9. Dawson, P. A., L. M. Lukaszewski, P. E. Ells, C. C. Malbon, and D. L. Williams. 1989. Quantification and regulation of apolipoprotein E expression in rat Kupffer cells. *J. Lipid Res.* **30**: 403–413.
10. Newman, T. C., P. A. Dawson, L. L. Rudel, and D. L. Williams. 1985. Quantitation of apolipoprotein E messenger RNA in the liver and peripheral tissues of nonhuman primates. *J. Biol. Chem.* **260**: 2452–2457.
11. Prack, M. M., M. Nicosia, D. L. Williams, and J. Gwynne. 1991. Relationship between apolipoprotein E mRNA expression and tissue cholesterol content in rat adrenal gland. *J. Lipid Res.* **32**: 1611–1618.

12. Nicosia, M., M. M. Prack, and D. L. Williams. 1992. Differential regulation of apolipoprotein E mRNA in z. fasciculata cells of rat adrenal gland determined by in situ hybridization. *Mol. Endocrinol.* **6**: 288–298.
13. Williams, D. L., J. S. Wong, S. L. Wissig, and R. L. Hamilton. 1995. Cell surface “blanket” of apolipoprotein E on rat adrenocortical cells. *J. Lipid Res.* **36**: 745–758.
14. Prack, M. M., G. H. Rothblat, S. K. Erickson, M. E. Reyland, and D. L. Williams. 1994. Apolipoprotein E expression in Y1 adrenal cells is associated with increased intracellular cholesterol content and reduced free cholesterol efflux. *Biochemistry.* **33**: 5049–5055.
15. Reyland, M. E., J. T. Gwynne, P. Forgez, M. M. Prack, and D. L. Williams. 1991. Expression of the human apoE gene suppresses steroidogenesis in mouse Y1 adrenal cells. *Proc. Natl. Acad. Sci. USA.* **88**: 2375–2379.
16. Swarnakar, S., M. E. Reyland, J. Deng, S. Azhar, and D. L. Williams. 1998. Selective uptake of low density lipoprotein-cholesteryl ester is enhanced by inducible apolipoprotein E expression in cultured mouse adrenocortical cells. *J. Biol. Chem.* **273**: 12140–12147.
17. Piedrahita, J. A., S. H. Zhang, J. R. Hagaman, P. M. Oliver, and N. Maeda. 1992. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc. Natl. Acad. Sci. USA.* **89**: 4471–4475.
18. Plump, A. S., S. K. Erickson, W. Weng, J. S. Partin, J. L. Breslow, and D. L. Williams. 1996. Apolipoprotein A-I is required for cholesteryl ester accumulation in steroidogenic cells and for normal adrenal steroid production. *J. Clin. Invest.* **97**: 2660–2671.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
20. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34**: 1345–1353.
21. Erickson, S. K., S. R. Lear, M. A. Barker, and T. A. Musliner. 1990. Regulation of cholesterol metabolism in the ethionine induced premalignant rat liver. *J. Lipid Res.* **31**: 933–945.
22. Voyno-Yasenetskaya, T. A., L. G. Dobbs, S. K. Erickson, and R. L. Hamilton. 1993. Low density lipoprotein- and high density lipoprotein-mediated signal transduction and exocytosis in alveolar type II cells. *Proc. Natl. Acad. Sci. USA.* **90**: 4256–4260.
23. Temel, R. E., B. Trigatti, R. B. DeMattos, S. Azhar, M. Krieger, and D. L. Williams. 1997. Scavenger receptor B, type I (SR-BI) is the major route for the delivery of high density lipoprotein cholesterol to the steroidogenic pathway in cultured mouse adrenocortical cells. *Proc. Natl. Acad. Sci. USA.* **94**: 13600–13605.
24. Feingold, K.R., P.M. Elias, M. Mao-Qiang, M. Fartasch, S. H. Zhang, and N. Maeda. 1995. Apolipoprotein E deficiency leads to cutaneous foam cell formation in mice. *Journal of Investigative Dermatology.* **104**: 246–250.
25. Kuipers, F., J. M. van Ree, M. H. Hofker, H. Wolters, V. In't, R. Havinga, R. J. Vonk, H. M. Princen, and L. M. Havekes. 1996. Altered lipid metabolism in apolipoprotein E-deficient mice does not affect cholesterol balance across the liver. *Hepatology.* **24**: 241–247.
26. Reyland, M. E., M. M. Prack, and D. L. Williams. 1992. Increased expression of protein kinase C in Y1 cells which express apolipoprotein E decreases basal steroidogenesis by inhibiting expression of P450-cholesterol side chain cleavage enzyme mRNA. *J. Biol. Chem.* **267**: 17935–17942.
27. Reyland, M. E., and D. L. Williams. 1991. Suppression of cAMP-mediated signal transduction in mouse adrenocortical cells which express apolipoprotein E. *J. Biol. Chem.* **266**: 21099–21194.
28. Raber, J., S. F. Akana, S. Bhatnagar, M. F. Dallman, D. Wong, and L. Mucke. 2000. Hypothalamic-pituitary-adrenal dysfunction in Apoe (–/–) mice: possible role in behavioral and metabolic alterations. *J. Neurosci.* **20**: 2064–2071.
29. Swarnakar, S., J. Beers, D. K. Strickland, S. Azhar, and D. L. Williams. 2001. The apolipoprotein E-dependent low density lipoprotein cholesteryl ester selective uptake pathway in murine adrenocortical cells involves chondroitin sulfate proteoglycans and an α -2-macroglobulin receptor. *J. Biol. Chem.* **276**: 21121–21128.
30. Glass, C., R. C. Pittman, M. Civen, and D. Steinberg. 1985. Uptake of high-density lipoprotein-associated apoprotein A-I and cholesterol esters by 16 tissues of the rat *in Vivo* and by adrenal cells and hepatocytes *in Vitro*. *J. Biol. Chem.* **260**: 744–750.